# ENZYMES IN ONTOGENESIS (ORTHOPTERA)

## XVIII. Esterases in the Grasshopper Egg [1]

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The respiratory quotients obtained for the grasshopper egg during all except the first few days of its development are characteristically those of an organism metabolizing fat (Bodine, 1929; Boell, 1935). Slifer (1930) has shown that the amount of fat in the egg measured in terms of fatty acid after saponification decreases during the phases of active development. The determination of the amounts of "lipoidal" substance that form the centripetal layer when a saline extract of these eggs is centrifuged also demonstrates a decrease in volume during embryonic growth (Bodine et al., 1939). Further, the potency of this "lipoidal" layer to activate the proenzyme, protyrosinase, varies in a different fashion than its change in volume (Bodine et al., 1939). These facts add an interest to the study of the types and activities of lipolytic enzymes present in the egg of the grasshopper (*Melanoplus differentialis*) during its embryogeny.

### Materials and Methods

Grasshopper eggs were collected daily and kept at 25° C. either in the pods or separated upon damp sand within covered glass dishes. Under these conditions the eggs go into diapause within a month (Slifer, 1931). This block in development was interrupted by keeping the eggs at 5° C. for three months and then transferring them to 25° C., at which temperature they hatched in 18 days. The eggs for experiments were washed, sorted, and sterilized with 70 per cent alcohol for ten minutes (eggs 0 and 5 days of age were not treated with alcohol), rinsed and ground in a glass mortar. The ground eggs were made up to designated volume in a glycine-NaOH buffer mixture. This egg brei was centrifuged and the lipoidal or centripetal layer removed, as were the shell fragments (*A* and *C* layer of Bodine and Allen, Fig. 1, 1938). The remainder was made up to volume with the buffer mixture. Removal of the *A* and *C* layers did not alter the enzyme activity.

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Amounts of the extract were added to a 50 cc. Erlenmeyer flask containing substrate and allowed to stand at 25° C. unless otherwise noted. Varying concentrations of enzyme and substrate were used, the total volume of the reaction mixture being 6 cc. After a period of time, the reaction was stopped with 25 cc. of a 2 per cent phenol solution and the mixture titrated with 0.05 N HCl until methyl red, used as an indicator, turned pink. Although the H ion concentration changed during the experiments, the addition of the phenol in buffer brought the pH back to the alkaline side and the HCl titre then was a measure of the NaOH neutralized during the reaction and was equivalent to the acid formed. The amount of acid thus formed is considered a measure of the rate of hydrolysis and an index of the amount of enzyme present. The equivalents of acid produced are not strictly rate values in the case of tributyrinase (Bodansky, 1937). Because of the difficulty in determining how much of the substrate was properly emulsified, a more accurate measure of rate was not practicable. Controls were duplicates of the experimentals with the exception that the enzyme extract was heated at 100° C. for 5 minutes. Reaction mixtures containing no substrate or enzyme were also tested and gave values equal to those of the control. Shaking the flasks during the reaction period did not change the rate of the hydrolysis.

The buffer mixture contained 0.1 N glycine and 0.1 N NaOH in the ratio of 9 to 1, 15 per cent glycerol, and enough NaCl to make the solution 0.17 M with respect to NaCl. The addition of salt was necessary to prevent precipitation of proteins in the extract. The phenol was dissolved in the glycine-NaOH buffer and was never used after it had acquired a brownish tinge. The methyl butyrate (Eastman Kodak), 2 per cent by weight, and the tributyrin (Eastman Kodak) and olive oil, 4 per cent by weight, were made up in the buffer containing glycerol and NaCl. Previous workers have experienced difficulty in making up tributyrin solutions which gave consistent results. Seventy milligrams of a commercial dispersing agent (Daxad No. 11) [2] per 100 cc. of solution will stabilize a tributyrin emulsion.

The method was checked using known amounts of butyric acid in place of the lipid in the protocol and the probable errors of the means of ten determinations at four concentrations between 0 and $2 \times 10^{-4}$ M butyric acid were less than 2 per cent of the mean in every instance. The amount of hydrolysis of an excess of methyl butyrate (3 cc. of 2 per cent) increases linearly with extracts of one to thirty diapause eggs. The amount of acid produced by one cc. of an extract (20 eggs per cc.) was linear with time for 4 hours. When tributyrin was used as a sub-

[2] Furnished by Dewey and Almy Chemical Co.

strate, an extract of 2 eggs (diapause) would produce as much acid in 2 hours as an extract of 30 eggs would produce from methyl butyrate. The amount of acid produced from 3 cc. of 4 per cent tributyrin was proportional to the concentration between 1 to 5 eggs per cc. The reaction on tributyrin was linear with time only for the first hour. In making the following determinations, 1 cc. of an extract containing 20 eggs per cc. with 3 cc. of 2 per cent methyl butyrate in a reaction period of 2 hours and 1 cc. of an extract containing 2 eggs per cc. with 3 cc. of 4 per cent tributyrin in a reaction period of one hour at 25° C. were used as test reactions.

In a number of experiments direct titrations of reaction mixtures were made with 0.05 N NaOH to determine the extent of hydrolysis. In these the pH was first adjusted by the addition of acid or alkali and titrations made to maintain this H-ion concentration. A Leeds and Northrop pH meter with a glass electrode was used in these titrations. The time course of the reactions under these conditions at steady pH values between 4.5 and 8.0 was similar to that when the method described above was used.

## Experimental

Enzymes are present in the grasshopper egg which will catalyze the hydrolysis of methyl butyrate and tributyrin but not olive oil. The enzymes are designated as methyl butyrinase and tributyrinase respectively in the following discussion although other substrates may be attacked by these enzymes. According to the nomenclature of Oppenheimer (1936), both are esterases; the one more specifically a lipase since it splits a glycerol ester of the fatty acid. The amounts of the two lipolytic enzymes vary independently during the development of the grasshopper egg (Fig. 1).[3] The methyl butyrinase activity is high at the start of development and then drops markedly between the tenth and fifteenth day. A steady level is then maintained during the diapause or inactive stage. Upon resumption of development a slow decline in activity takes place. Tributyrinase action, however, remains at the same level during prediapause and diapause and then drops rapidly in post-diapause development. An extract from a single grinding was used on both substrates in each of the ten determinations represented by the averages in the figure.

Two types of experiments were used to determine the relative amounts of the lipolytic enzymes being studied in the embryo and yolk

[3] In preliminary work (Carlson, 1940) the enzyme extracts used were so concentrated that the changes in activity were obscured.

constituents of the egg. Early prediapause (6-day) eggs were irradiated at 1000 roentgens, which is known to prevent the embryo from developing but to have no visible effect on other constituents of the egg (Evans, 1936).[4] The oxygen consumption of eggs treated in this manner decreases until the time of diapause. The $O_2$ uptake is low during diapause, and when the diapause is broken the oxygen uptake increases for the first two days and then remains constant (Bodine,

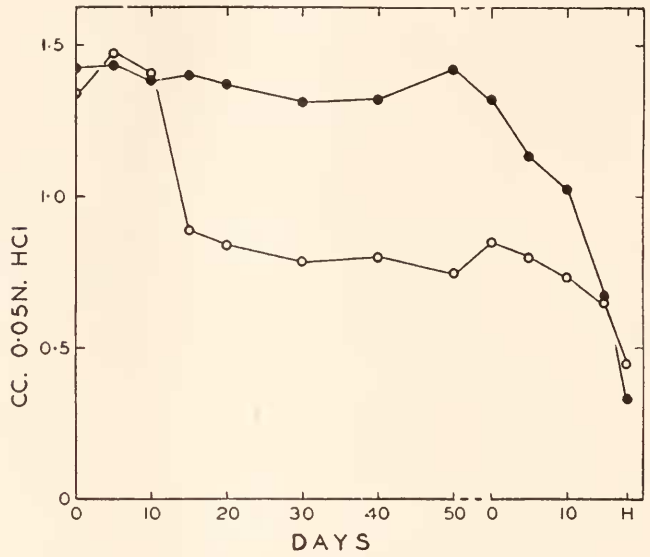


Fig. 1. Average esterase activity of five lots of eggs at each of the developmental ages shown. Ordinate, the equivalents of acid produced by hydrolysis of the esters in cc. of 0.05 N HCl; abscissa, time in days at 25° C. since laying followed by the time in days at 25° C. after termination of the diapause by exposure to 5° C. for three months. Open circles, the activity of an extract of 20 eggs in two hours with methyl butyrate; closed circles, that of an extract of two eggs in one hour with tributyrin as a substrate.

Carlson, and Ray, 1940). No significant difference could be shown between the enzyme content of the irradiated eggs and that of the controls. Determinations were carried out for 30 days after the irradiation.

In postdiapause, the embryos could be dissected from the egg and determinations made of the lipase content of the embryo and other egg constituents. The dissections were carried out in the buffer mixture. The embryos were freed from as much adhering yolk as possible and transferred with a minimum of fluid to a mortar and ground with a

[4] Dr. T. C. Evans irradiated the eggs for the author.

small amount of sand. The remaining yolk and shells were also ground and used with the dissection fluid in the determinations. There was some difficulty in freeing the embryos of yolk, but in all cases this was done as completely as possible. The amounts of enzyme in embryo plus yolk, etc. were always less than those of the whole eggs. The lipolytic enzymes seem to be associated with the yolk or its derivatives until just previous to hatching (Table I). The yolk removed in later stages of development usually included parts of the gut that could not be adequately separated. The presence of some esterase in the 5-day post-diapause embryos is attributed to the fact that the yolk and embryos were especially hard to separate at this stage.

Although the time course of reactions was similar at different pH values, the extent of hydrolysis of tributyrin was markedly affected.

TABLE I

| Days Post-diapause | Embryo | Yolk etc. | Substrate |
|---|---|---|---|
| | *Per cent* | *Per cent* | |
| 0 | 0 | 100 | Tributyrin |
| 5 | 19.3 | 80.7 | |
| 10 | 0 | 100 | |
| 15 | 0 | 100 | |
| 18 | 100 | 0 | |
| 0 | 0 | 100 | Methyl butyrate |
| 5 | 20.3 | 79.7 | |
| 10 | 8.7 | 91.3 | |
| 15 | 0 | 100 | |
| 18 | 100 | 0 | |

Reactions were carried out at pH 4.5, 5.0, 6.0, 7.0, and 7.5 by titrating frequently with 0.05 N NaOH. In Fig. 2, curve *A* shows the total amount of alkali used in this procedure over a one-hour period with the enzyme from two eggs reacting with 3 cc. of 4 per cent tributyrin. Similarly, curve *B* shows the result of experiments using the extract of 20 eggs with 3 cc. of 2 per cent methyl butyrate. The pH for maximum tributyrinase activity is at 6 while the H-ion concentration affects the methyl butyrinase activity to a lesser degree. This dissimilarity in the effect of pH on the activity of the enzymes studied is one of several differences noted. No explanation of the difference in the values for the rate of methyl butyrate hydrolysis when the pH is kept constant and when it is allowed to change is at hand.

The effect of heat treatment on the enzyme extract as well as its effect on the amount of hydrolysis was determined. In the former case

the lipolytic activity of the extract was affected differently for the two substrates. The activities of extracts were tested at 25° C. after ten-minute exposures to temperatures between 25 and 85° C. The ability to split methyl butyrate was diminished by temperatures higher than 55° C. while tributyrinase activity was unchanged after exposures to 65° C. (Fig. 3).

When the reaction mixtures were kept at temperatures varying from 0° to 45° C., the amount of hydrolysis of the two substrates differed in

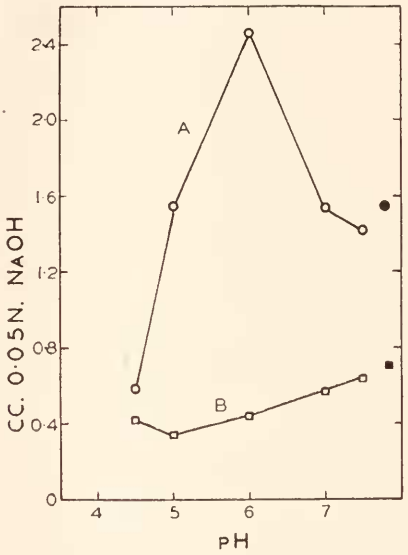


Fig. 2. The amount of hydrolysis at various H-ion concentrations. The reactions were kept at the pH noted by continuous titrations with 0.05 N NaOH. ○, the hydrolysis of tributyrin by an extract from two eggs over a period of one hour; □, methyl butyrate split by an extract of 20 eggs at the end of 2 hours. Solid symbols represent amount of acid formed when the mixture was allowed to react over the total time. Four to seven experiments averaged in each point. Reactions at room temperature.

a striking manner. The hydrolysis of methyl butyrate increased with temperatures up to 45° C. (Fig. 4*A*) and between 0° and 35° a $\mu$ value of 5700 calories was obtained (Fig. 4*B*). The $Q_{10}$ over the corresponding range averaged 1.4. The tributyrinase activity showed a maximum at 25° C. with a decrease on either side of this temperature (Fig. 4*A*). The $Q_{10}$ value between 5 and 15° C. is 1.97; between 15 and 25°, 1.47, using the amount of acid produced per unit time as a rate value. The values shown in the figure were obtained using an extract made in the

following manner: the eggs were ground and diluted to a volume so that the concentration was 40 eggs per cc. When this was allowed to stand a considerable precipitate was formed. This was centrifuged off and the supernatant fluid diluted to a volume corresponding to 20 eggs per cc. This resulting extract still retained its tributyrinase activity, but the methyl butyrinase reaction was reduced to one-sixth to one-fifth of that of an extract prepared in the usual manner. Falk and Sugiura (1915) were able to separate esterase and lipase materials in the castor bean, the one soluble in distilled water, the other in NaCl solution. The temperature relationship to activity of the enzyme is similar to that reported by Fiessinger and Gajdos (1936) working with an extract of the larva of *Galleria mellonella*. Their extract showed maximum activ-

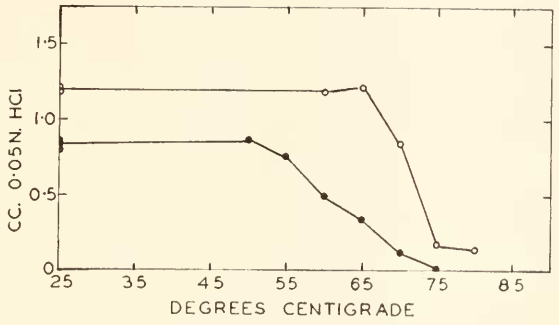


FIG. 3. The effect of temperature on the activity of the enzyme extract. Ordinate, equivalents of acid produced in cc. of 0.05 N HCl in one hour for tributyrinase and two hours for methyl butyrinase at 25° C.; abscissa, temperature in °C. to which the extract was exposed for 10 minutes. Closed circles, the hydrolysis of methyl butyrate; open circles, the hydrolysis of tributyrin. Methyl butyrinase from 20 eggs; tributyrinase from 2 eggs.

ity between 18° and 25° C. and declined at temperatures above or below this range.

Most observations concerning esterase activity indicate that the calcium ion, sodium oleate and albumin accelerate the activity of the enzymes. This is not found to be the case in extracts of the grasshopper egg. Sodium oleate reduces the lipolytic activity of the preparations used in these experiments. Calcium chloride has no effect on the enzyme but counteracts in part the effect of sodium oleate (Table II). Neither of these substances has any effect in stabilizing the pH. Albumin was not used since the extract was rich in protein. Attempts to show hydrolysis of olive oil with addition of sodium oleate and the calcium ion to the egg extracts at 25° C. and 35° C. were without success.

Various esterases are affected differently by such compounds as

phenol, quinine, atoxyl and sodium fluoride (Falk, 1924; Oppenheimer, 1936). Curiously, extracts from pancreas, liver and kidney are inhibited in their action on tributyrin in a diverse manner by quinine and atoxyl (Falk, 1924). The effect of 0.5 per cent phenol, NaF and quinine were tested on the esterases obtained from the grasshopper egg.

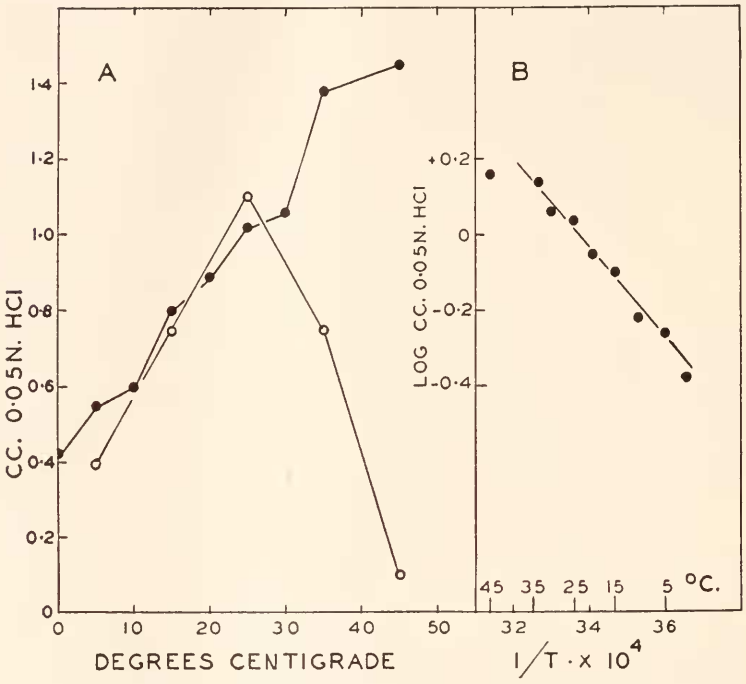


FIG. 4. The amount of hydrolysis of methyl butyrate and tributyrin at different temperatures. In *A*, the ordinate gives the equivalents in cc. of 0.05 N HCl; the abscissa, the temperature at which the reaction took place. ○, tributyrinase reaction using an extract of 2 eggs with 4 per cent tributyrin for 1 hour; ●, methyl butyrinase from 20 eggs reacting with 3 cc. of 2 per cent methyl butyrate for 2 hours. *B* shows the data for methyl butyrinase plotted as log concentration of HCl, ordinate, versus the reciprocal of the absolute temperature × $10^4$, abscissa. The points are average values of ten determinations at each temperature. The straight line in *B* is fitted by the method of least squares. The $\mu$ value between 0 and 35° C. is approximately 5700 calories. For further description see text.

The results are summarized in Table III. Both methyl butyrinase and tributyrinase are inhibited by quinine and NaF. Only tributyrinase is inhibited by the 0.5 per cent phenol; methyl butyrinase activity is stimulated. Two per cent phenol will completely block both reactions. Fiessinger and Gajdos (1936), in studies on the esterase obtained from the larva of *Galleria mellonella,* found their enzyme extract unaffected by

phenol and quinine and strongly inhibited by NaF in the same concentrations as noted above with tributyrin as a substrate.

## Discussion

The expectation that the grasshopper egg contains an enzyme capable of hydrolyzing triglycerides of higher fatty acids was perhaps based on a fortuitous assumption. The presence of such an enzyme in an animal metabolizing fat as the R.Q. indicates (Bodine, 1929; Boell, 1935) and consuming 60.3 per cent of its initial store of fats during development (Slifer, 1930) seemed highly probable. No evidence for this enzyme could be elicited using the methods described. The activity

Table II

| | 0.05N HCl (in cc.) | | | |
|---|---|---|---|---|
| Control | Control + NaOl 0.2 cc. −0.4% | Control + NaOl + $CaCl_2$ 0.4 cc. −2% | Control + $CaCl_2$ | Substrate |
| 1.19 | 0.03 | 0.21 | 0.74 | Tributyrin |
| 0.52 | 0.06 | 0.23 | 0.33 | Methyl butyrate |

Table III

| | 0.05N HCl (in cc.) | | | |
|---|---|---|---|---|
| Control | 0.5% Phenol | 0.5% NaF | 0.5% Quinine HCl | Substrate |
| 1.06 | 0.53 | 0.50 | 0.19 | Tributyrin |
| 0.77 | 1.64 | 0.19 | 0.42 | Methyl butyrate |

on the esters of the lower fatty acid (butyric) was, however, quite high during early stages of development. A summary of the data concerning the lipids of the grasshopper eggs is of interest. The fat in the egg of *Melanoplus differentialis* is liquid at room temperature (fusion point, 26.2° C.); in *Chortophaga viridifasciata* the fat is solid (fusion point, 39.4° C.) (Slifer, 1930). The former insect spends the winter as an egg, the latter as a nymph. The iodine number of the fats is the same in both animals (135 to 140) (Slifer, 1932). The low melting point in the winter eggs may possibly be due to the higher proportion of short chain fatty acids. This is the explanation of the liquid fat of the aphid, *Pemphigus*, which contains glycerides of butyric, caprylic and lauric acids (Timon-David, 1927–28). The presence of monoesters

rather than glycerol triesters might give similar results. The data concerning the enzymes present in the grasshopper egg lend credence to the assumption that the lower fatty acids are present in the egg. Slifer (1930) has shown that the total fat (measured after saponification by a method for higher fatty acids) decreases only slightly during prediapause (9.7 per cent), yet the volume of the lipoidal layer as measured by Bodine et al. (1939) decreases 32.5 per cent in the same period. Slifer (1930) found a loss of 50 per cent in postdiapause, the volume determinations, 42.5 per cent. The amount of fatty acids in a diapause egg is approximately 8 per cent of the wet weight of the egg (Slifer, 1930) as compared to an amount of lipid equal to 3½ per cent of the wet weight of the egg obtained by the centrifuge separation. The fat obtained by this latter method is a mixture of esters (probably glycerol) which contains $C_{12}$ to $C_{18}$ fatty acids (Allen, T. H., personal communication). Experiments to determine the hydrolysis of the lipid separated by centrifuging and also lipids extracted from the egg brei with petrol ether showed demonstrable amounts of hydrolysis after a 24-hour period only in the case of the latter. This might well be due to the existence of esters and acids in equilibrium.

The relative amounts of hydrolysis in these two enzymatic reactions cannot be quantitatively compared with the activity of esterases from other sources. In general it seems evident that the enzymes are relatively concentrated in the grasshopper egg, since experiments described with other esterases involve periods of four hours and upwards at 37° C. to produce enough acid to be measured. Fiessinger and Gajdos (1936) found that the tributyrinase from the larva of *Galleria mellonella* was much more active than that from human blood serum (ca. 10 times). They also could demonstrate no reaction with olive oil as a substrate.

The two lipolytic enzymes possess strikingly different physical and chemical properties as evidenced by the independent change in potency during development, the inactivation by heat, the effect of temperature on the rate of hydrolysis, the possibility of separating the two enzymes, and the difference in effect of the inhibitors used. Curiously, the tributyrinase, *per se,* is less sensitive to heat treatment than methyl butyrinase yet more susceptible to temperature in the presence of its substrate. This may be due to a reversal of the heat inactivation in the former case.

The evidence indicates the lipolytic enzymes in the grasshopper are present in greatest quantities at the time the egg is laid. From these high levels at the time of least differentiation in the egg the enzymes decrease in amount during development or differentiation (Fig. 1). A change in the amounts of esterase in the egg of the trout (*Salmo fario*) was observed by Falk and co-workers in a careful and detailed study of

this material. Methyl butyrate was not hydrolyzed by the esterase from the unfertilized egg, but the hydrolysis was accomplished by eggs 35 or more days after fertilization. Methyl and ethyl acetates were easily hydrolyzed by the egg but steadily less so as development proceeded; ethyl butyrate showed a reverse effect. The value of esterase action generally was high in immature eggs, small in mature eggs, increasing with development (see Needham, 1931, for summary). In the work of Falk et al. cited here no data are given for the esters of the long chain fatty acids. In the grasshopper egg the decline in the activity of the monobutyrinase after the tenth day of prediapause development occurs somewhat later than the decline in potency of the natural activator (presumably a lipid) of protyrosinase (Bodine et al., 1939). It is possible that some of the substances serving as activators are monoesters of fatty acids and that these are utilized rapidly in early development. Subsequent to this period the amount of monobutyrinase falls. However, the explanation of this effect suggested by Bodine and Carlson (1940) seems more tenable. The decline in the amounts of both enzymes studied during post-diapause development seems correlated with the rapid disappearance of yolk. The possibility that these enzymes may be found in the serosa has not been excluded in these experiments, yet the major part seems to be contained in the yolk and probably is incorporated into the midgut after its absorption. This conforms to the evidence of Stuart (1935) that the yolk cells become part of the midgut just previous to hatching. The cells of the intestinal tract then "inherit" these enzymes from the yolk. Other hydrolytic enzymes may come to be in the gut of the adult in a similar manner.

## Summary

1. Glycerol extracts of the grasshopper egg (*Melanoplus differentialis*) have been tested for hydrolytic activity on methyl butyrate, tributyrin and olive oil during various stages in the development of the egg. The ability to hydrolyze methyl butyrate is high when the egg is laid; this value declines between the tenth and fifteenth day of development, remains constant during diapause and slowly declines again during the post-diapause period. The action of extracts on tributyrin is much stronger, remains constant from the time of laying until the cessation of the diapause and then declines markedly. No action on olive oil could be demonstrated.

2. Optimum activity in hydrolysis of tributyrin is at pH 6; the activity of the enzyme reacting with methyl butyrate is only slightly affected by changes in the H-ion concentration.

3. Temperature affected the methyl butyrinase and tributyrinase activity in a different manner. Exposure to temperatures above 55° C. depressed the activity of the former while the activity of the latter persisted to 65° C.

4. The hydrolytic action on tributyrin increased with temperature between 5° and 25° C. and declined at higher temperatures. Methyl butyrinase activity increased with temperature between 0° and 45° C.

5. The esterases seemed to be associated with the yolk until just before hatching.

6. The effect of sodium oleate, calcium ion and various inhibitors of lipolytic enzymes on the extracts used were determined.

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